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Zoological Research

Phylogeny of *melanogaster* Species Group Inferred from ND4L and ND4 Genes

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Abstract: The relationships within *Drosophila melanogaster* species group are controversial from morphology, chromosomes and DNA sequences. This study utilises a molecular approach aimed at uncovering the phylogenetic relationships among 33 taxa representing 8 subgroups of *D. melanogaster* species groups. Mitocondrial ND4L-ND4 was sequenced in the all 8 subgroups covering a wide geographic area. MP and Bayesian analysis produced an identical tree topology with relatively strong support in most nodes. It reveals that the *melanogaster* species group clustered in three main lineages: 1) *montium* subgroup; 2) *ananassae* subgroup; 3) Oriental subgroups (*melanogaster*, *ficsphila*, *eugracilis*, *elegans*, *suzukii* and *takahashii*). The *montium* subgroup branched off first, followed by the *ananassae* subgroup. In the third lineage, *melanogaster* is the most divergent subgroup followed by *ficsphila*, *eugracilis*, *elegans* in that order. The *suzukii* and *takahashii* sister subgroups are the last to branch off.

Key words: Phylogenetic; *melanogaster* species group; Mitochondrial; ND4L-ND4

以 ND4L 和 ND4 基因为标记探讨黑腹果蝇种组的系统发育关系

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摘要: 多年来的形态学、染色体组学以及 DNA 序列几个方面的研究均没有很好地阐明黑腹果蝇种组内的系统发育关系。本实验测定了 33 个样品的 ND4 和 31 个样品的 ND4L 基因序列, 以 *D. obscuroides* 为外群, 用最大简约法和 Bayesian 法分别构建进化树。结果表明两种方法构建的拓扑结构一致, 而且大部分支系的支持率较高。整个黑腹果蝇种组分成三大谱系: 1) *montium* 种亚组; 2) *ananassae* 种亚组; 3) Oriental 种亚组(*melanogaster*、*ficsphila*、*eugracilis*、*elegans*、*suzukii*、*takahashii*), *montium* 是最早分化的种亚组。在第三谱系中, *melanogaster* 分化得最早; 然后依次是 *ficsphila*, *eugracilis*, *elegans*; *suzukii* 与 *takahashii* 为姐妹种亚组, 最后分化。

关键词: 系统发育关系; 黑腹果蝇种组; 线粒体; ND4L-ND4
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The wide geographical distribution and the large number of species make the *melanogaster* species group an attractive system for evolution studies. The *melanogaster* species group is currently thought consisted 174 species, including a number of unclassified species that are too poorly known for the affinities to be apparent. Most of species are distributed in Afrotropical or Oriental regions (Ashburner et al, 1984; Bock & Wheeler, 1972; Lachaise et al, 1988; Schawaroch, 2002; Kastanis et al, 2003). The use of morphological characters, including the structure of male genitalia (which seems to be very variable for some species), is

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usually enough for the identification of genera and species but are still insufficient to infer precise phylogeny. The utility of morphological characters in determining the relationships within *melanogaster* species group is limited (Hsu, 1949; Okada, 1954; Bock & Wheeler, 1972) and only 12% ~ 50% of the current species in the group are known (Schawaroch, 2002). Ashburner et al (1984) using chromosomes and morphology, discerned three lineages: ① *ananassae* subgroup; ② *montium* subgroup; ③ a lineage comprised of the *elegans*, *eugracilis*, *ficusphila*, *melanogaster*, *suzukii* and *takahashii* subgroups.

Except for the result of Inomata et al (1997), which *eugracilis* subgroup was close to *ananassae* subgroup and the other subgroups divided into two main lineages inferred from *Amy multigenes*, other molecular data are accorded with the opinion of three main lineages: one represented by the *ananassae* subgroup, the second by the *montium* subgroup, and the third comprising the *elegans*, *eugracilis*, *ficusphila*, *melanogaster*, *suzukii*, and *takahashii* subgroups. However, there are different opinions about how these lineages are interrelated. Yang et al (2004) suggested the *montium* subgroup was the ancestral subgroup followed by the *ananassae* subgroup based on spacer region of H2A-H2B histone gene. Schawaroch (2002) proposed *ananassae* and *montium* subgroups as sister subgroups based on alcohol dehydrogenase, hunchback and cytochrome oxidase Ⅱ sequences. Others agreed with the opinion that the *ananassae* was the ancestral subgroup followed by *montium* subgroup. The Oriental lineage (the third lineage) was particularly interesting because of its morphological diversity and close proximity to *D. melanogaster*. But up to now, phylogeny of Oriental subgroups is still most controversial, even the integrated analysis of the molecular data and previous morphological studies.

The mitochondrial genes have proven to be a powerful tool in phylogenetic studies. Kastanis (2003) discussed the phylogenetic relationship of *melanogaster* species group based on mtDNA of 1.7 kb. However, this fragment of mitochondrial DNA included rRNA, tRNA and protein coding genes, which had different structural and functional constraints. These must make cladogram not robust. Steinbachs et al (2001) investigated the efficiency of 15 distinct genes of mitochondrian (13 protein-coding and 2 rRNA) in recovering a known *Drosophila* genealogy (*Drosophila melanogaster* subgroup), and concluded that ND4 recovered the true genealogy most efficiently. Their result suggested ND4 was a good genetic marker in a close-related group of species.

In this study, we selected two protein-coding genes (ND4L-ND4) to reconstruct the phylogeny of *melanogaster* species group in both subgroup and species levels.

1 Materials and Methods

1.1 Fly species

Most specimens were collected in China. Information about the name, locality, and Genbank accession numbers of the specimens was shown in Tab. 1.

1.2 DNA extraction, amplification, and sequencing

One adult flies were homogenized and suspended in a 50 mmol/L NaCl, 30 mmol/L Tris-HCl (pH 8.0), and 200 mmol/L EDTA solution, and the genomic DNA was extracted with phenol/chloroform, precipitated with ethanol, and suspended in TE solution. PCR amplification of the ND4L-ND4 fragment gene was made by using the following primer (modified from Yu et al, 1999): ND4F, 5′-ATCACTAACACCACAAATTAG-3′; ND4R, 5′-TTTGATTTACAAGACCAATG-3′. The cycling profile for ND4L-ND4 was 95 ℃ for 3 min, 35 cycles of 94 ℃ for 60 s, 53 ℃ for 60 s, and extension at 72 ℃ for 1.5 min, and a final extension period of 72 ℃ for 20 min. The PCR products were directly ligated into pGEM-T Easy vectors and the positive clones were screened out. For sequencing, we have used the same PCR primer plus the external primer-21M13. The consensus nucleotide sequence is obtained for two different clones from at least two sequencing reactions.

1.3 Sequence alignment and phylogenetic analysis

Computer alignments were implemented in CLUSTAL W program (Thompson et al, 1994), moreover, the result of alignments was manually adjusted.

In parsimony analysis, all character are unordered and weighted equally. MP trees are constructed in PAUP (Swofford, 1998) by running the heuristic search with TBR branch swapping, 100 random addition sequence replications, and non-parameter bootstrap re-sampling procedures were applied to get the coincidence of MP trees.

Bayesian analysis were performed in MrBayes 2.01 (Huelsenbeck & Ronquist, 2001) with general-time-reversible + gamma + invariants (GTR + G + I) model of sequence evolution and four Markov chain Monte Carlo (MCMC) sampling to assess phylogenetic relationships. We set the parameters in MrBayes as fol-

lowing: nst = 6, rate = gamma, basefreq = estimate, generations = 1 000 000, and the posterion probability and branches of the phylogeny are summed by burnin = 500 and contype = allcompat.

D. obscuroides from *obscura* species group was defined as an out-group in the phylogenetic analysis.

Tab. 1 List of *Drosophla melanogaster* species considered in analysis

Subgroup	Species	Collection location	GenBank accession No.
montium	*D. auraria*	Hubei, China	AY958400
	D. triauraria	Henan, China	AY958419
	D. lini	Yunnan, China	AY958411
	D. leontia	Yunnan, China	AY958410
	D. barbarae	Guangdong, China	AY958402
	D. baimaii	Hainan, China	AY958401
	*D. trapezifrons*1	Hubei, China	AY958413
	*D. trapezifrons*2	Guangdong, China	AY958418
	*D. trapezifrons*3	Guangxi, Chin	AY958397
	D. parvula	Hainan, China	AY958416
	D. jambulina	Hainan, China	AY958409
	D. costricta	Guangdong, China	AY958405
ananassae	*D. ananassae*	Hainan, China	AY958399
	D. malerkotliana	Guangxi, China	AY958398
	D. parabipectinata	Hainan, China	AY958415
	D. bipectinata	Hainan, China	AY958404
suzukii	*D. suzukii*	Guangdong, China	AY958423
	D. biarmipes	Hainan, China	AY958403
	D. pulchrella	Guangdong, China	AY958421
	D. sp. *chayu*	Xizang, China	AY958417
	D. lucipennis	Guangdong, China	AY958412
takahashii	*D. takahashii*	Hainan, China	AY958425
	D. sp. *curveaedeagus*	Xizang, China	AY958422
	D. prostipennis	Guangdong, China	AY958420
	D. trilutea	Hubei, China	AY958424
melanogaster	*D. melanogaster* *		NC_001709
	D. yakuba *		X03240
	D. simulans *		AF 200834
	D. mauritana *		AF 200830
	D. sechellia *		AF 200832
ficusphila	*D. ficusphila*	Guangdong, China	AY958408
eugracilis	*D. eugracilis*	Hainan, China	AY958407
elegans	*D. elegans*	Hainan, China	AY958406
obscura	*D. obscuroides*	Xizang, China	AY958414

The sequences of species marked with * were from Genbank.

2 Results

2.1 Data character

ND4L sequences of *D. malerkotliana* and *D. trapezifrons* 3 are not determined. Between two genes, two additional nucleotide (AT) insertions are found in *takahashii* subgroup (*D. takahashii*, *D.* sp. *curveaedeagus*, *D. prostipennis*, *D. trilutea*) and part members of *suzukii* subgroup (*D. suzukii*, *D. pulchrella*). We conclude that ND4L genes of these species end in complete termination codon TAA and others end in TA.

ND4L and ND4 genes have a strong A and T bias respectively as other insect mitochondrial sequences (ND4: A = 31.52%, T = 47.24%, C = 7.92%, G = 13.32%; ND4L: A = 51.46%, T = 31.92 %, C = 10.50 %, G = 6.12 %; Cyt*b*: A = 31.03 %, T = 42.78%, C = 13.31%, G = 12.88%).

No length variation is observed among all species for ND4L and ND4 genes. The ND4 gene is 1 339 nucleotides long, 474 (35.4%) of which are variable and 316 (23.6%) are parsimony informative (without the out-group). The ND4L gene is 290 nucleotides long, 65 (22.4%) of which are variable and 42 (14.5%) are parsimony informative. Moriyama & Powell (*1997*) have proposed that the unusually low divergence found

in ND4L was probably due to selective constraints on the secondary structure of the transcript.

2.2 Phylogenetic analyses

Fig. 1 shows the consensus tree from MP and Bayesian analysis based on ND4. Moreover, there is no variety of topology based on ND4L-ND4 integrated analysis. We recognize three main lineage according to the result, comprised of: (Ⅰ) *montium* subgroup; (Ⅱ) *ananassae* subgroup; (Ⅲ) the Oriental subgroups: *melanogaster*, *ficsphila*, *eugracilis*, *elegans*, *suzukii* and *takahashii* subgroups. *D*. sp. *chayu* and *D. lucipennis* (*suzukii* subgroup) make a clade with *elegans* subgroup (BP = 68, PP = 96), other subgroups are apparent monophyletic. *montium* is first branched off followed by *ananassae* subgroup. In the Oriental subgroups, *melanogaster* appears to be the most basal subgroup, *ficsphila*, *eugracilis*, *elegans* branch off in that order, *suzukii* and *takahashii* are placed as sister groups in a weakly support.

ND4 makes a good resolve for the phylogenetic relationships of closely related species. High bootstrap support is for all nodes within subgroups. In *montium* subgroup, the unclassified species, *D. baimaii* branches off firstly, the others are divided into two main monophyletic clade with high bootstrap values in both analyses (PP > 85): (Ⅰ) *D. trapezifrons*, *D. costricta*, and *auraria* complex. (Ⅱ) *D. parvula*, *kikkawai* complex and *jambulina* complex.

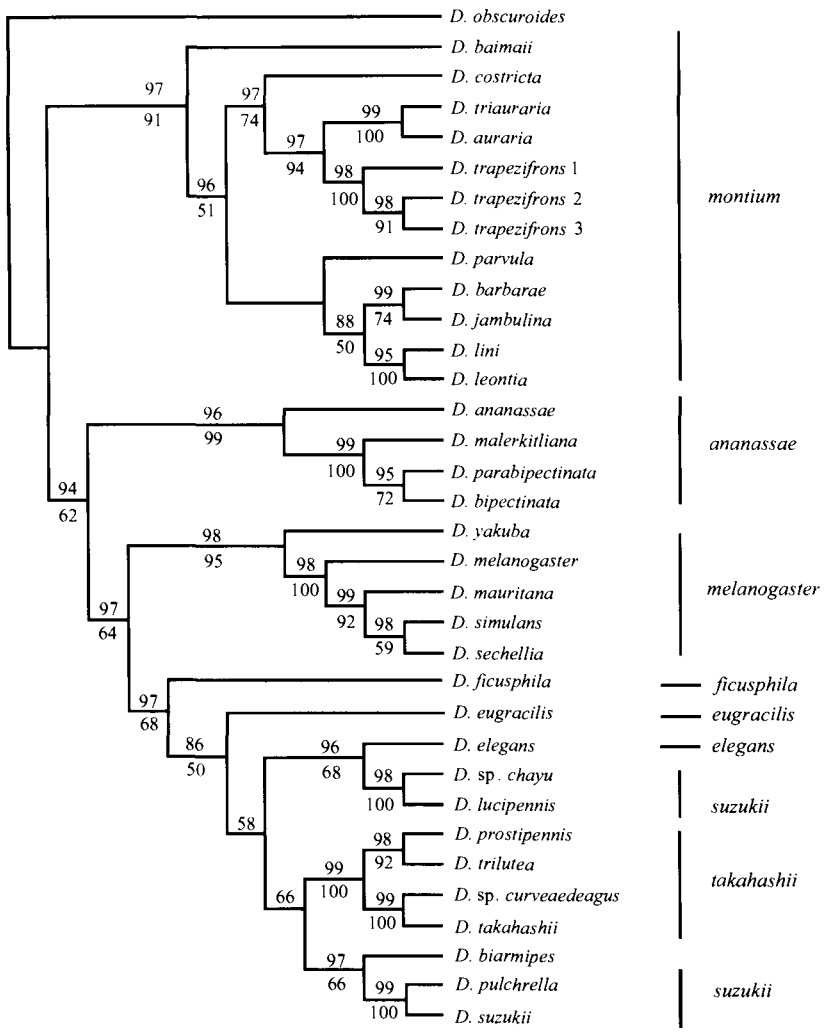

Fig. 1 Phylogenetic relationships of *melanogastr* species group based on ND4 using Maximum parsimony and Bayesina analysis

Numbers under the branches are bootstrap percentage values for clades supported above a 50% bootstrap value in MP analysis; numbers above the branches are posterior probabilities in Bayesian analysis with MCMC algorithm.

3 Discussion

It is worth pointing out that in most previous studies the *melanogaster* species group was represented by a small mount of species. In present study, we obtain 33 taxa from eight subgroups.

Our results are congruent to the result of Yang et al (2004) who analyzed the spacer region of the *histone* gene H2A-H2B from 36 species of *Drosophila melanogaster* species group. *montium* subgroup first branches off followed by *ananassae* subgroup, Oriental subgroups (*melanogaster*, *ficsphila*, *eugracilis*, *elegans*, *suzukii* and *takahashii*) form a well monophyletic group branches off in the end. It is contrary to precious hypothesis based on morphological and chromosomal data (Bock, 1980; Ashburner et al, 1984; Lemeunier et al, 1986) and most of molecular data (Pélandakis & Solignac, 1993; Lage et al, 1996; Inomata et al, 1997; Clark et al, 1998; Goto & Kimura, 2001).

Previous molecular data have suggested different relationships among species subgroups within the Oriental lineage. The two most comprehensive studies, in terms of both taxon sampling and the amount of data, are those of Schawaroch (2002) and Kopp & True (2002). The former, based on alcohol dehydrogenase (*Adh*), hunchback and cytochrome oxidase Ⅱ (*Co* Ⅱ) sequences, supports a close relationship between *D. elegans* and *D. lucipennis*, as in our study. *D. ficusphila* was most basal species in the Oriental subgroups followed by *D. elegans-D. lucipennis*. *eugracilis* was placed as a sister taxon to the *melanogaster* subgroup. Unfortunately, many nodes in the phylogeny of Schawaroch (2002) have low bootstrap support (< 50%). The study of Kopp & True (2002), based on 28*S* ribosomal RNA, cytochrome oxidase subunit 1 sequence, alpha-amylase gene, glycerol-3-phosphate dehydrogenase, dynein heavy chain gene and fragment of the mitochondrial ND1. *eugracilis* branched off first followed by *ficusphila*, *melanogaster* subgroup was the closest to *takahashii-suzukii*.

A sister group relationship between *takahashii* and *suzukii* subgroups has been inferred on numerous occasions using morphological and chromosomal data (Ashburner et al, 1984; Lemeunier et al, 1986), and DNA sequences data (Inomata et al, 1997; Pélandakis & Solignac, 1993; Goto & Kimura, 2001; Clark et al, 1998). Both phylogeny and sequence character of ND4L (two additional insertions) support the previous result.

In our phylogeny, *suzukii* subgroup is polyphyletic. Three species traditionally ascribed to this subgroup, *D. suzukii*, *D. pulchrella*, and *D. biarmipes* do form a monophyletic group. *D. lucipennis* and *D.* sp. *chayu*, are very distant from this clade, as a sister taxa to the *elegans* subgroup. The results matched the hypothesis of Kopp & True (2002), which proposed the polyphyly of *suzukii* subgroup. The *elegans-D. lucipennis-D.* sp. *chayu* clade clusters in turn with *takahashii-szuzukii* clade.

In our phylogeny, *melanogaster* subgroup is in the basal position of Oriental subgroups (BP = 64, PP = 97), which is compatible with the result of *CO* Ⅰ and *Gpdh* (Goto & Kimura, 2001). The relationships of *ficusphila* and *eugracilis* are still obscure. Pélandakis et al (1991), Pélandakis & Solignac (1993) suggested that *eugracilis* sister to *melanogaster* based on the rDNA sequences data, Inomata et al (1997) assumed *eugracilis* was close to *ananassae*, Yang et al (2004) assumed *eugracilis* as sister group to *melanogaster*. In Fig. 1, *eugracilis* and *ficusphila* are supported as sister group, which is consistent with the opinion of Kopp & True (2002).

The *montium* subgroup was the largest in the *Drosophila melanogaster* species group and comprised 81 known species. It was distributed throughout Northeat Asia (Japan, Korea and China), the South Pacific Islands (Borea, Sumatra, Java and Australia) and Indian and Afrotropical area. In the present experiment, our phylogenetic hypothesis supports that the *jambulina* complex was closer to the *kikkawai* complex than to the *auraria* complex, which was consistent with previous studies (Ohnishi et al, 1983; Ohnishi & Watanabe, 1984; Kim et al, 1989, 1993; Zhang et al, 2003). Among different geographic populations, high genetic differentiation and polymorphism have been found in *D. trapezifrons*, populations from Hubei, Guangdong, and Guangxi are distinct morphologically and have genetically differentiated to the level of subspecies or even semispecies. In the clade Ⅰ, *D. trapezifrons* and *D. costricta* (unclassified species) and the *auraria* complex show a close phylogenetic relationship with high confidence values (BP = 74, PP = 97). In addition, they are similar morphologically to the *auraria* complex. *D. barbarae* is very curious and the previous analyses always yield conflicting results (Schawaroch, 2002). It was originally suggested on the basis of the morphology of its male genitalia that *D. barbarae* belonged to *kikkawai* complex (Lemeunier et al, 1986). Later, Kim et al (1989) assigned

this specie to the *jambulina* complex on the basis of cross experiments. In our result, *D. barbarae* shows a closed phylogenetic relationship with *D. jambulina* (BP = 75, PP = 94) and should be assigned to the *jambulina* complex.

The *ananassae* subgroup was widespread from Africa across Asia. Our results agree with previously proposed classifications based on morphology (Bock, 1980). *D. ananassae* lacking dimorphism in abdominal coloration was quite different from the other members. *D. malerktoliana*, *D. parabipectianana*, *D. bipectianana*, and *D. pseudoana* have been termed "*bipectianana* complex", which had a similarities in sex-combs. In our phylogeny, *D. ananassae* is the most basal member in this subgroup (boostrap = 98%), *D. malerktoliana*, *D. parabipectianana* and *D. bipectianana* make a group with high bootstrap value consistently to the result of Pélandakis & Solignac (1993).

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